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Beta Thalassaemia Mutations in Malays: A Simplified Cost-effective Strategy To Identify the Mutations

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ABSTRACT

Background/Aims: Beta (β)- thalassaemia is a public health problem in Malaysia. The carrier rate is estimated to be 4.5% by micro-mapping studies particularly among Malays who comprise 53.5% of the population in Malaysia (1). The common diagnostic method in Malaysia for mutation detection is by amplification refractory mutation system (ARMS). It allows single mutation detection in each reaction but is labour intensive and time consuming when many mutations need to be identified. The purpose of this study was to develop a diagnostic tool for effective mutation detection of beta thalassaemia in Malay patients and compare its efficacy with ARMS-PCR, the current method in use. **Methods:** Reverse dot blot hybridization (RDBH) technique was incorporated in the development of two strip assays [RDBH-Strip M(6) and RDBH-Strip C(6)] to identify common beta thalassaemia mutations in the Malays. The panels of selected mutations were based on the mutation frequencies in Malaysia reported in previous studies. RDBH-Strip M(6) was applied as step 1 and RDBH-Strip C(6) was applied as step 2 for unidentified mutations. The strips were validated with the gold standard method, ARMS-PCR. **Results:** One hundred and thirty seven Malay patients with 274 alleles were studied. In Step 1 mutation detection, 238 alleles (86.9%) were identified in 119 of patients by RDBH-Strip M(6). Step 2 resulted in a further detection of 20 alleles in another 10 patients by RDBH-Strip C(6). The combination of both strips resulted in the identification of 258 alleles in 129 (94.6%) of 137 Malay patients. The strip assays were 100% sensitive and specific when compared with ARMS-PCR method. **Conclusion:** Two strip assays utilising the RDBH technique were developed to identify common β-thalassaemia mutations in Malays. The RDBH Strip M(6) identified 86.9% of the mutations and the RDBJ-Strip C (6) detected further 7.3% alleles. This two step strategy was found to be rapid and cost effective for the direct diagnosis of β-thalassaemia mutations in the Malays. The remaining unidentified mutations would require DNA sequencing. It can serve as a specific molecular diagnostic tool for effective diagnosis of β-thalassaemia mutations in this ethnic group.

Keywords: β-thalassaemia, Malays, Malaysia, Reverse dot blot hybridisation, ARMS-PCR

INTRODUCTION

The demographics of Malaysia are represented by the multiple ethnic groups that exist in the country. Malaysia's population as of July 2010 is estimated to be 28,946,227. Malays and other Bumiputera groups make up 65% of the population, Chinese 26%, Indians 8% and other unlisted groups 1% [1]. Beta-thalassaemia is a public health problem in Malaysia. About 4.5% of the population in Malaysia have been identified as heterozygous carriers for beta thalassaemia, particularly among 53.5% of the population who are Malays [2, 3].

Beta thalassaemia is one of the common genetic disorders worldwide with a defect in haemoglobin synthesis [4, 5]. Beta thalassaemia is extremely heterogeneous at the molecular level with more than 200 different mutations identified. The vast majority of the mutations are caused by point mutations with the exception of a few deletions [6, 7, 8, 9]. However, each ethnic group has its own characteristic set of beta-thalassaemia mutations with common and some rare mutations.

In the Malays, three common β-thalassaemia mutations IVS I-5(G C), IVS I-1(G→T) and CD 17(A→T) were reported to account for 81.25% of the β-thalassaemia mutations among transfusion dependent patients [10]. Some rare mutations reported were CD8/9, CAP+1 and poly A [2, 11]. The common structural haemoglobin variants HbE [CD26(CAG→AAG)] and Hb Malay [CD19(A→G)] accounted for 29% and 7.1% of mutations respectively [2, 11]. Concomitant inheritance of HbE with β-thalassaemia is also a common scenario amongst the Malay population. The

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most common β-thalassaemia allele in HbE individuals is IVS I-5 (G→C) [10, 11]. The type of β-thalassaemia allele determines the severity of the phenotypes in HbE/β-thalassaemia patients.

Current methods for definitive diagnosis of thalassaemia are mainly DNA-based techniques. These methods are traditionally separated into two categories: direct and indirect mutation detection. Direct mutation detection detects the presence or absence of known mutations such as in amplification refractory mutation system polymerase chain reaction (ARMS-PCR) [12, 13], dot blot hybridization [14, 15, 16] and restriction fragment length polymorphism [17, 18] . These methods are easily applied for the detection of known single mutations but are tedious when many mutations need to be identified [16, 19] . Indirect mutation detection screens a specific region of genes to identify the variations within that region such as in denaturing gradient gel electrophoresis (DGGE) [13, 20] , denaturing high performance liquid chromatography (dHPLC) [21] and single-strand conformation polymorphism (SSCP) [22]. These are efficient methods for screening unknown mutations but it is mandatory to apply an alternative method to confirm the identified mutation to prevent misdiagnosis [13] . These latter methods require special apparatus and skilful technical support [13] .

The current method for the detection of beta thalassaemia mutations in Malaysia is ARMS-PCR [3, 9, 11] . It allows single mutation detection in each reaction and is tedious, labour-intensive and time-consuming when a number of mutations need to be identified.

To institute a comprehensive thalassaemia control program in Malaysia, an effective and practical diagnostic tool needs to be developed to identify common mutations simultaneously and accurately. In this study, we explored a more advantageous DNA practical diagnostic tool, reverse dot blot hybridization (RDBH) for rapid, specific and sensitive molecular detection of beta thalassaemia mutations in the Malay population of Malaysia.

METHOD

Approval was obtained from the ethical committee of Faculty of Medicine and Health Sciences, University Putra Malaysia (UPM) and ethical committee of University of Malaya Medical centre (UMMC) prior to the commencement of the study.

Samples

One hundred and thirty seven (137) venous whole blood samples collected in ethylenediamine tetra-acetic acid (EDTA) as an anticoagulant from Malays were provided with permission by Institute of Medical Research (IMR) in Kuala Lumpur for this study. The patient samples were presumptively identified as beta thalassaemia through the BHES protocol (B: blood counts, blood film, H: high performance liquid chromatography, E: electrophoresis, S: Stability) [23, 24] .

Normal control samples were obtained from twenty biomedical students, Universiti Putra Malaysia with normal haemoglobin level, normal red cell indices and normal haemoglobin subtypes. Positive control samples were provided by Dr. Law Hai Yang of Kandang Kerbau (KK) Women's and Children's Hospital in Singapore and Prof. Dr. Mary Anne Tan of University Malaya Medical Centre (UMMC) in which 12 mutations were previously identified and consisting: CD 26 (CAG→AAG), IVS I-5 (G→C), IVS I -1 (G→T), CD 19 (A→G), CD 8/9(+G), CAP+1 (A→C), CD 41/42 (-TTCT), IVS II-654 (C→T), CD 17 (A→T), -28 (A→G), -29(A→G) and CD 71/72 (+A).

DNA analysis

Genomic DNA was extracted from leucocytes in peripheral whole blood using QIAamp DNA midi kit (Qiagen GmbH, Germany). Quality of DNA was determined by electrophoresis using 1.0% pre-stained ethidium bromide (2 mg/ml) agarose gel at 10 volts/cm for 30 minutes in 1X TAE buffer (tris-acetate-EDTA). DNA was quantified using Nanodrop 1000 spectrophotometer (Thermo-scientific, Thermo Fisher Scientific Inc., Wilmington, U.S.A.).

RDBH-Strip assay

Two strip assays [RDBH-Strip M(6) and RDBH-Strip C(6)] were designed and developed specifically to identify six common mutations in Malays and Chinese-Malaysians according to the frequency of common β-thalassaemia mutation found. RDBH-Strip M(6) was designed for six common mutations in Malays [CD 26 (CAG→AAG), IVS I-5 (G→C), IVS I -1 (G→T), CD 19 (A→G), CD 8/9(+G) and CAP+1 (A→C)] while RDBH-Strip C(6) for another six common mutations in Chinese-Malaysians [CD 41/42 (-TTCT), IVS II-654 (C→T), CD 17 (A→T), -28 (A→G), -29(A→G) and CD 71/72 (+A)].

Detection using developed RDBH-strip assays involved the following five steps:

(i) Preparation of Strip

Biodyne C negative charged nylon membrane was gridded, labelled and cut. The membrane was washed with 0.1N HCl and activated with 10% EDAC (N'ethyl-3-(3-dimethylaminopropylcarbodi-imide).

(ii) Amplification of target DNA with the biotinylated primers to the beta-globin gene

Two sets of biotinylated primers [22] (Table 1) were used for amplification of DNA. Biotinylated primers set 1 [RDBC1, RDBC 2, RDBC 3] was amplified for analysis on RDBH-Strip M(6) while both biotinylated primers set 1 and set 2 [RDBC 4,RDBC 5, RDBC 6, RDBC 7] for RDBH-Strip C(6). Reaction was carried in 50 µl containing 200 ng of genomic DNA template, a mixture of 50 mM KCl, 10 mM Tris (pH 8.3), 2.5mM MgCl2, 200µM of mixture dNTP, 0.4 µM of each primer and 2.5 units of MaximaTM hot start taq DNA polymerase (Fermentas life science, California, USA). The cycling reaction was performed in a programmable thermal cycler (Takara PCR thermalcycler DiceTM, TP 600 gradient, Takara Bio Inc., Otsu, Shiga) at initial denaturation for 5 minutes at 94ºC, followed by 35 cycles at 94ºC for 1 minute, 55ºC for 30 seconds, 72ºC for 30 seconds and a final extension at 72ºC for 10 minutes. Five microlitres of each amplified product was analyzed using 1.5% gel electrophoresis in 1X TAE buffer at 90 volts for 30 minutes. Detection was carried out using agarose gel incorporated with ethidium bromide and visualized on an ultraviolet transilluminator (G:box bioimaging systems, Synoptics Ltd, Cambridge, UK).

Table 1. Sequences of biotinylated primer

ID	5' → 3' sequence (5' Biotin-)		Genbank ID: nucleotide	
RDBC 1	GTA CGG CTG TCA TCA CTT AGA CCT CA	Fw	HUMHBB 01317:62008-62033	
RDBC 2	TGC AGC TTG TCA CAG TGC AGC TCA CT	Rv	HUMHBB 01317:62609-62584	602bp
RDBC 3	GCT AGT GAA CAC AGT TGT GT	Rv	HUMHBB 01317:62169-62150	162bp
RDBC 4	GTG TAC ACA TAT TGA CCA AA	Fw	HUMHBB 01317:63088-63107	423bp
RDBC 5	AGC ACA CAG ACC AGC ACG TT	Rv	HUMHBB 01317:63510-63491	
RDBC 6	TCT GAT AGG CAC TGA CTC TC	Fw	HUMHBB 01317:62538-62377	340bp
RDBC 7	CCT ATG ACA TGA ACT TAA CC	Rv	HUMHBB 01317:62697-62678	

Fw: forward, Rv: reverse

(iii) Immobilisation of Allele-specific oligonucleotide (ASO)

Two sets of 5'-amine end bound wild type and mutant oligonucleotide probes as previously described [25, 26] from Bio Basic Inc, Markham Ontario, Canada were used for immobilization onto the negatively charged nylon membrane. The spotted strips were hybridised with the biotin-labelled PCR fragment.

(iv) Hybridisation of biotinylated amplification products to the oligo-immobilized strip assay

Hybridization buffer and all the biotinylated PCR amplified products were mixed and placed at 95°C water bath for 15 minutes. The prepared strips were then placed into the hot solution mix. RDBH-Strip M(6) was hybridised at 42°C while RDBH-Strip C(6) was hybridised at 45°C. After that, the strips were transferred to wash buffer (0.5xSSC, 0.1% SDS) and washed at 42°C by a shaker.

(v) Detection of specifically bound mutant and wild type alleles by visible enzymatic colour reaction

After the hybridisation, the probes were tagged with a non-radioactive enzyme, horse-peroxidase (HRP) through covalent binding for colour detection. The enzyme-oligonucleotide conjugate probes hybridised to the target DNA sequences and were detected through simple oxidation of colourless soluble chromagen to a blue insoluble product using 3,3',5,5'-Tetramethylbenzidine (TMB) substrate.

Two- step strategy in beta thalassaemia mutation detection

In view of possible historical links and inter-marriage between Malays and Chinese, the Malay patients were first tested by RDBH-Strip M(6) and followed by RDBH-Strip C(6) for unidentified mutations as described in the experimental flow chart in Figure 1. The developed strip assays were run in parallel with ARMS-PCR for validation.

ARMS-PCR

Validation was done by running ARMS-PCR in parallel for the detection of the 12 common beta thalassaemia mutations (CD 26 (CAG→AAG), IVS I-5 (G→C), IVS I-1 (G T), CD 19 (A→G), CD 8/9(+G), CAP+1 (A→C), CD 41/42 (-TTCT), IVS II-654 (C→T), CD 17 (A→T), -28 (A G), -29(A→G) and CD 71/72 (+A).

RESULTS

Amplification of biotinylated primers to the beta globin gene

Two amplicons (602bp and 162bp respectively) were displayed by successful amplification of biotinylated primers set 1 to the β-globin gene. Successful amplification of biotinylated primers set 2 to the β-globin gene demonstrated two amplicons (423 bp and 340 bp respectively).

Mutations identification with the RDBH-strip assay

The panel of common mutations immobilized on RDBH-Strip M(6) and RDBH-Strip C(6) are shown in the Figure 2. The presence of a particular sequence was detected by the appearance of blue-coloured dots on the membrane as target DNA hybridized only to probes that are complementary to sequences therein. The blue dot that occurred with a mutant probe was interpreted as having that particular mutation in the β-globin gene. The normal controls showed blue dots on the left lane as DNA hybridized only to probes complementary to normal sequences. The heterozygous patient showed dots to the particular mutant probe (right lane) and all other normal probes (left lane). In a compound heterozygous patient, dots appeared at two mutant probes and all normal probes. In a homozygous patient, only a single dot appeared in the mutant probe (right lane) but not on the wild type probe.

Mutations identification with the RDBH-strip assay in Malays

In mutation analysis for 137 Malays consisting of 274 alleles, RDBH-Strip M(6) was able to identify 238 alleles [119 patients (86.9%)]. In the second step, RDBH-Strip C(6) identified 20 alleles [10 patients (7.3%)] out of 36 alleles from 18 patients unidentified by RDBH-Strip M(6). By the combined use of both strips, RDBH-Strip M(6) and RDBH-Strip C(6), we were able to identify 94.2% of the mutations in Malays in this study. Sixteen alleles remained unidentified in 8 Malay patients. Samples from these patients are to be sent for DNA sequencing. The frequency of the mutations identified in 137 patients is demonstrated in Table 2 and the alleles of the mutations identified are demonstrated in Table 3. By using RDBH-Strip M(6), the most common β-thalassaemia allele found was CD 26 [79 (28.8%)], followed with IVS I-5 [34 (12.4%)], IVS I-1 [19 (6.9%)] and CD 19 [18 (6.6%)]. The least common β-thalassaemia allele identified was CD 8/9 [1 (0.4%) allele]. In the second step, 18 samples were tested by using RDBH-Strip C (6). The most common β-thalassaemia allele found was CD 41/42 [5 (1.8%)], followed with 2 alleles with CD 17, -29 and CD 71/72 (0.7%) respectively.

Table 2. Frequency of β-thalassaemia mutations identified in Malays using RDBH-Strip M(6) and RDBH-Strip C(6)

	Number (n)	Percentage (%)
1. β-thalassaemia trait:		
• IVS I-5/ wild type	21	15.3%
• IVS I-1/ wild type	11	8.0%
• CD 19/ wild type	10	7.3%
• CD 41/42/ wild type	5	3.6%
• CD 17/ wild type	2	1.5%
• -29 /wild type	2	1.5%
• CD 8/9 / wild type	1	0.7%
• CAP+1/ wild type	0	0%
2. Homozygous β-thalassaemia:		
• Homozygous CD 19 (CD 19/CD 19)	1	0.7%
• Homozygous CD 71/72	1	0.7%
3. Compound heterozygous β-thalassaemia:		
• IVS I-1/ CD 19	1	0.7%
4. Hb E trait :		
• CD 26/ wild type	44	32.1%

Continuation

Table 2. Frequency of β-thalassaemia mutations identified in Malays using RDBH-Strip M(6) and RDBH-Strip C(6)

	Number (n)	Percentage (%)
5. Homozygous Hb E :		
• CD 26/CD 26	5	3.6%
6. Compound heterozygous Hb E:		
• CD 26/ IVS I-5	13	9.5%
• CD 26/ IVS I-1	7	5.1%
• CD 26/CD 19	5	3.6%
7. Not identified	8	5.8%
Total	137	100%

Table 3. Frequency of alleles identified for β-thalassaemia mutations in Malays

	Number (n)	Percentage (%)
RDBH-Strip M(6)		
1. Wild type	87	31.8%
2. CD 26 (G to A)	79	28.8%
3. IVS I-5 (G to C)	34	12.4%
4. IVS I-1 (G to T)	19	6.9%
5. CD 19 (A to T)	18	6.6%
6. CD 8/9 (+G)	1	0.4%
7. CAP+1 (A to C)	0	0%
RDBH-Strip C(6)		
8. Wild type	9	3.3%
9. CD 41.42 (-TCTT)	5	1.8%
10. CD 17 (A to T)	2	0.7%
11. -29 (A to G)	2	0.7%
12. CD 71/72 (+A)	2	0.7%
13. IVS II-654 (C to T)	0	0%
14. -28 (A to G)	0	0%
15. Not identified	16	5.8%
Total	274	100%

Validation of RDBH-Strip assay with ARMS-PCR

The mutations identified were validated with the standard gold method, ARMS-PCR. The mutations identified from the developed strip assay were found to be in complete concordance with ARMS-PCR. No false positive or false negative was found. The specificity and sensitivity were 100%.

DISCUSSION

In this study, two strip assays designated as RDBH-Strip M(6) and RDBH-Strip C(6) were developed specifically for Malays and Chinese-Malaysians respectively. Each strip can identify six mutations simultaneously.

The Malay race consists of two groupings, the Proto Malays and the Malay Polynesians. Originally, Malaysia was occupied by the Proto Malays who in Peninsula Malaysia are the indigenous population termed `Orang Asli'. These Proto Malays migrated to Peninsula Malaya in the ice-age, when the continental plate and the islands were connected by land. The most numerous of the Orang Asli are called Negritos and are related to native Papuans in Indonesia and Papua New Guinea. A red cell membrane disorder, hereditary ovalostomatocytosis which acts as a protection against malaria is found in these populations that are related by ancestry. The 2nd major wave of migration, the Malay Polynesians were from Champa (circa 500 AD till 1500AD), an area in central Asia from the Yunnan province. Other migrants to Peninsula Malaya originate from Arabia or India (from the west) and China (from the east). In our earlier studies on beta-thalassaemia mutations in the Malays, the most common mutation in the Chinese CD41-42 (-TCTT) was also seen in the Malays although IVS1-5(G→C) remains the most common in this group. In view of possible inter-marriage between the 2 major ethnic groups, Malays and Chinese-Malaysians, a second step was introduced for mutation detection. RDBH-Strip C(6) was applied as second step for mutation detection in samples unidentified by RDBH-Strip M(6) in Malays. This innovative 2-step strategy was able to identify 94.2% of the mutations out of the total of 137 Malay patients with 86.9% identified by RDBH-Strip M(6) and 7.3% by RDBH-Strip C(6). The strips encompassing specifically only 6 mutations each were easier to be optimized than a strip with numerous mutations to cover all mutations present in southeast Asia. This strategy makes it easy for use in any laboratory wanting to do mutation identification. In addition, it was found to be efficient, less time consuming and more cost effective than the ARMS-PCR method currently in use.

A heterogeneous pool of gene mutations were noted in the Malay patients studied. The most common β-thalassaemia alleles found were the IVS I-5 [34 (12.4%) alleles], followed by IVS I-1 [19 (6.9%) alleles], CD 41/42 [5 (1.8%) alleles], and two (0.7%) alleles were found with CD 17, -29 and CD 71/72 respectively. The least common β-thalassaemia identified was CD 8/9 [one (0.4%) allele]. The mutations identified are in keeping with common mutations described in previous studies [10, 11, 27] .

The two most common haemoglobin variants seen were HbE [79 (28.8%)] and Hb Malay [18(6.6%)] out of the total 274 alleles. Forty-four (32.1%) individuals were identified as heterozygous for HbE and five (3.6%) individuals were homozygous among the 137 Malay patients. The second most common haemoglobin variant was Hb Malay, with ten (7.3%) patients heterozygous for Hb Malay, one (0.7%) patient homozygous and compound heterozygous for Hb Malay respectively.

Interactions of HbE with β-thalassaemia alleles were observed in this study. The variable clinical phenotypes observed in HbE/β-thalassaemia may have resulted from the inheritance of either a mild (β^+) or severe (β^0) β-thalassaemia allele together with the HbE gene [28] . In this study, compound heterozygous for HbE/ IVS I-5 was found as most common [13 (9.5%)] followed by HbE/ IVS I-1 [seven (5.1%)] while the least common was HbE/CD 19 [five (3.6%)].

The developed strip assay was found to be reliable, together with high precision and high sensitivity (100% sensitivity and specificity). It was able to provide the same result in repeated tests using this strip assay and able to identify the mutant or wild type precisely without false positive results. The developed RDBH-Strip assay was significantly superior when compared to the standard gold method, ARMS-PCR. The developed RDBH-strip assay in this study was able to identify six mutations unlike the ARMS-PCR which is only able to identify one mutation at a time with the wild type and mutant reactions done separately. Therefore, the developed strip assay is fast in mutation analysis compared to ARMS-PCR. ARMS-PCR requires several days of turnaround time since each sample requires performing several PCR reactions to detect different mutations. This takes up much genomic DNA, is time-consuming and labour-intensive. In contrast, the developed RDBH-strip assay required an estimated five hours to test each strip for six mutations simultaneously in one PCR reaction. This indicates a high throughput compared to ARMS-PCR. In documentation aspect, the developed RDBH-Strip assay is more convenient than ARMS-PCR as it is self-documented and easy to store. In the interpretation of results, the developed strip assay is easier in differentiating heterozygous and homozygous alleles with wild type and mutant probes situated at left-right columns compared to ARMS-PCR where analysis occurs in different gels. In addition, the developed RDBH-strip assay uses streptavidin-HRP conjugate with TMB substrate for detection while ARMS-PCR uses ethidium bromide which is carcinogenic for detection. Furthermore ARMS-PCR is not cost effective. Twelve ARMS-PCR reactions are required to identify six mutations for which the cost is RM180. The developed strip assay is able to identify a similar number of mutations at an estimated cost of RM55.

CONCLUSION

The practical PCR based 2-step strategy with the developed RDBH-strip assay was found to be rapid, accurate and cost-effective for the identification of beta-thalassaemia mutations in Malays. It is recommended as a good substitute for the ARMS-PCR method.

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